Appendix B6

Lyticase-based cell lysis protocol of β-Galactosidase assay for 96 well plates

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1 Media and solutions

Complete minimal (CM) dropout medium without leucine

Per 1 liter:
1.26 g dropout powder (a mix of essential amino acid, Sigma)
6.7 g yeast nitrogen base (YNB) without amino acids or ammonium sulfate (Difco)
5 g ammonium sulfate
10 g D-Glucose
Autoclave for 15 min.

Z buffer

Per 1 liter:
16.1 g Na_2HPO_4 7 H_2O (60 mM final)
5.5 g NaH_2PO_4 H_2O (40 mM final)
0.75 g KCl (10 mM final)
0.246 g $MgSO_4$ $7H_2O$ (1 mM final)
2.7 ml β-mercaptoethanol (50 mM final)
Adjust to pH 7.0. Do not autoclave

Lyticase 10X stock solution (1µg/µl)

Per 10 ml:
10 mg lyticase (Sigma)
1ml potassium phosphate buffer 1 M, pH 7.5
0.2 ml NaCl 5 M
5 ml glycerol,
Complete to 10 ml with H_2O
Store at –20°C.

ONPG substrate

Per 100 ml:
400 mg ONPG in potassium phosphate buffer 0.1 M pH 7.0
Filter sterilized and stored at –20 °C.

2 Cell growth

2.1 Growth on solid media

Yeast from glycerol stock were streaked on CM plates plus leucine and incubated at 30°C. Single colonies may be seen after 24 h but require 48 h before they can be picked for growth in liquid media.

2.2 Growth in liquid media

Four independent colonies were picked and inoculated in 4 Erlenmeyer flasks containing 5 to 10 ml CM medium plus leucine (or appropriate selective medium). The volume should not exceed 1/5 of the total flask volume. Yeast cells are incubated at 30°C in a shaking incubator at 300 rpm (the optimal speed depends on the orbital radius of the shaker) for 24 to 36 hours.

Note 1: It is important for all glassware to be detergent-free
Note 2: Other material than Erlenmeyer flask can be used (50 ml Falcon tube, for example).

2.3 Determination of cell density

The density of cells in liquid culture can be determined spectrophotometrically by measuring its optical density (OD) at 600 nm. For reliable measurements, cultures should be diluted such that the OD_{600} is <1 (generally 1/10 dilution in H_2O). In this range, 0.1 OD_{600} unit corresponds to ~$7x10^6$ cells/ml with our spectrophotometer. It is advisable to calibrate the spectrophotometer by graphing OD_{600} as a function of the cell density that has been determined by direct counting in a hemacytometer or titering by spreading on CM plates for viable colonies.

3 Assay for β-Galactosidase in 96-well plates

This protocol describes a rapid, quantitative assay of β-Galactosidase activity of recombinant yeasts for estrogen receptor (ER) in liquid culture. Yeast cells are lysed by hypo-osmotic shock after wall cell digestion by lyticase.

3.1 cell suspension seeding in conic bottom 96-well plate

After determination of the cell density, the culture is diluted in CM plus leucine to obtain 0.5-0.6 OD_{600} (see **2.3** for calibration of the spectrophotometer).For one 96 well culture plate 12-15 ml (in 15 ml Falcon tube) of cell suspension is required. Generally 3 plates from 3 independent colonies are used in parallel. 100 µl of yeast suspension are distributed in each well with multi-channels pipette (8 or 12 channel) excepted the first row (1 to 12 row or A to H column) which receives 200 µl of cell suspension. The first row containing 200 µl of yeast suspension is reserved for dilution of test compounds.

3.2 dilution of test compounds

Two µl of 100X test compounds (dissolved in ethanol or DMSO) are added in each well of the first row (200 µl of cell suspension) to obtain the first concentration (1/100 dilution of the stock solution). Mix gently 4 to 5 times the suspension with the multi-channels pipette. Dump 100 µl in the second row of the plate (1/2 dilution). Repeat this process for the other rows and eliminate the 100 µl in excess for the last row.

Note: Another procedure can be used. This consists of in making in separated tubes a series of dilution of 50 or 100 fold concentrated compounds in DMSO. One or two µl of each dilution are distributed in each well before cell suspension distribution. It is important in this process to use

DMSO as solvent to avoid evaporation of solutions in the plate during the distribution of compounds in each well. Ethanol is too volatile for this procedure.

3.3 Yeast cell incubation with test compounds

Plates are incubated for 4 hours at 30°C without shaking in an appropriated humidified incubator. Plates must be covered to avoid evaporation and they must not be directly exposed to the air flow of the incubator. This point is particularly important.

3.4 cell suspension recovering

After four hours of treatment, plates are centrifuged for 5min in an appropriated rotor for 96-well plates at 1500 rpm max (higher speed can break the plate). The supernatant is eliminated from each well using a vacuum pump.

At this step, cell pellets can be stored at –20 to –80°C for several days.

At this step, since the residual medium volume is low, the pellet can be gently resuspended using a vortex. This should be performed carefully to avoid cross-contamination between wells. This step is very important for an efficient action of lyticase in the next step.

4 Protocol of lyticase cell lysis

4.1 cell lysis

Dilute 10 time stock solution of lyticase (1µg/µl) in Z buffer solution. Fifty µl (5 µg) of lyticase in Z buffer is added in each well for 30 min at room temperature (20-22°C). Do not use vortex at this step. The temperature is very important for an optimal lysis during 30 min so that an incubator should be used if the room temperature was higher than 22°C.

After incubation with lyticase, 100 µl of 0.01% triton X100 solution in H_2O is added in each well and incubated for 15 min at room temperature. This hypo-osmotic shock is sufficient for a complete lysis of yeast cells but a freezing/thaw cycle at –80°C can be performed to obtain complete cell lysis if necessary (**see 4.2**).

Note: Do not freeze the extracts for a long time at this step to avoid apparition of protein aggregation. These protein aggregates will be pelleted with cell debris during the centrifugation in the next step and up to 90% of soluble proteins as well as β-Galactosidase activity can be lost after 3 days at –80°C.

4.2 β-Galactosidase assay and protein quantification

Plates are centrifuged in an appropriated rotor for 96-well plates at 1500 rpm max for 5 to 10 min. At this step, the pellets appear opalescent and more spread out than cell pellets; this indicates a good lysis of yeast cells. If the pellets appear very white and compact, this indicates a bad lysis of cells, In this case, perform freezing/thawing cycle after re-suspension of the pellets.

Aliquots of 100 µl of supernatants are transferred in flat bottom 96-well microtitration plates and 50 µl of ONPG substrate are added in each well. The enzymatic reaction is performed at 30°C for 1 to 2 hours for rtER and 30 mn to 1 hour for hER. Read the OD_{405} when the yellow color appears sufficient (0.8-1 OD_{405} for the maximal induction); stop the reaction by adding 50 µl Na_2CO_3 1M and read the plate after 5 minutes of equilibration.

During β-Galactosidase reaction, perform protein quantification. For this, 10 or 20 µl of soluble extract are added to 200 µl of Coomasie blue reagent. Add BSA standard: 0, 1, 2, 4, 6, 8 µg BSA per well and calculate the quantity of proteins in 150 µl of the total volume of lysis solution. The same correction is realized for OD_{405}.

Determine the β-Galactosidase units defined as OD_{405}/mg protein/minute of enzymatic reaction.

Note1: The accuracy of protein quantification is important for reproducibility of measurements of β-Galactosidase activity between independent experiments.

Note2: The definition of the β-Galactosidase is an arbitrary personal unit, not an official unit.